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(54) Title: METHODS, COMPOSITIONS AND SCREENING ASSAYS RELATING TO AUTOIMMUNE DISEASE

(57) Abstract

Disclosed herein is the discovery that the insertion of a retroviral transposon of the *ETn* family into the *fas* apoptosis gene coding region underlies the *lpr/lpr* rodent model of systemic autoimmune disease. These results establish a link between endogenous retrovirus expression and autoimmune disease. The present invention embodies both novel nucleic acid probes and new screening assays for use in the identification of agents for the treatment of autoimmune and lymphoproliferative diseases. The invention also contemplates new therapeutic strategies for autoimmune disease involving modulating the expression of retroviruses associated with tolerance-related or lymphocyte activation genes.

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DESCRIPTION

METHODS, COMPOSITIONS AND SCREENING ASSAYS RELATING TO AUTOIMMUNE DISEASE

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates generally to immunology and autoimmunity. Disclosed is the discovery that the *fas* apoptosis gene, a gene of importance in tolerance induction, is rendered dysfunctional by a retroviral insert. The invention describes novel nucleic acid probes and screening assays for use in the identification of candidate substances for the treatment of autoimmune and lymphoproliferative diseases.

2. Description of Related Art

The phenomenon of autoimmunity underlies the development of several common diseases, including many arthritis-linked disorders such as rheumatoid arthritis and systemic lupus erythematosus. Rheumatoid arthritis is a chronic, immune-mediated inflammatory disease that primarily affects the joints and their supporting structures, although the disease sometimes involves other organs and tissues such as the eyes, lungs, heart and skin (Harris, 1990). This common disease produces profound morbidity and excess mortality in an estimated 1% of the population (Lawrence *et al.*, 1989).

Despite progress in understanding how the immune system works, the pathogenesis of autoimmune diseases still remains obscure. As a consequence, better treatment of these diseases remains elusive. Although life expectancy has improved, due principally to

ancillary services such as antibiotics, blood banks and control of disease complications, treatment remains non-specific relying upon corticosteroids and immunosuppressive drugs which have dangerous and life threatening side-effects. Significant progress clearly needs to be made in understanding the etiology and pathogenesis of autoimmune disease.

It is important to distinguish between autoimmunity and autoimmune disease. The former is often benign whereas the latter is potentially fatal. Autoimmunity is the presence of serum autoantibodies and is a normal consequence of aging, it is readily inducible by drugs or infectious agents, and is potentially reversible in that it disappears when the offending drug or agent is removed or eradicated. Autoimmune disease results from activation of self-reactive T and B cells which, following stimulation by genetic or environmental triggers, cause actual tissue damage.

The mechanism of autoimmune disease is not well understood, but such diseases may arise because of a defect in removing immune cells that react against the organism itself. The generation of self-reactive lymphocytes is a consequence of the immune system designed for immunoresponses against foreign antigens. However, in a normal immune system, the self-reactive cells generated are usually removed, either by clonal deletion or inactivation on interaction with body antigens, thereby triggering cell death.

Autoimmune disease is dependent upon at least four factors. Two of the major factors are genetic and viral. A third factor is endocrine, based on the ability of estrogen to promote autoimmune disease, whereas androgen acts as a natural immunosuppressive agent. These are physiological modulatory effects of sex hormones acting

on normal immune responses, and explain the marked female predominance of autoimmune disease. The fourth factor is psychoneuroimmunologic (i.e. the influence of stress and neurochemicals on the immune response). A common feature of these four factors is the ability to affect gene expression. Genes influence autoimmune disease through their classic role as immune response regulators acting primarily through the major histocompatibility complex (MHC), but perhaps also through immunoglobulin (Ig) and T cell receptors (TCR).

Attempts to develop effective pharmacological agents for use in treating autoimmune diseases have not met with significant success, partly because the few animal models used for autoimmune studies are equally poorly understood. Some success has been achieved in studying autoreactive immunoglobulins which have been studied in transgenic mice expressing an antibody to murine red blood cells (Okamoto *et al.*, 1992). These mice, unlike normal mice, have large numbers of self reactive B lymphocytes so that it is relatively easy to follow the selection of these cells *in vivo*.

Another model system is the MRL-*lpr/lpr* mouse, a model of systemic autoimmune disease in which intrinsic defects of intrathymic T cell development have been noted (Cohen & Eisenberg, 1991; Zhou *et al.*, 1991; 1992). MRL-*lpr/lpr* mice have been reported to express endogenous retroviruses (Kreig *et al.*, 1991), but the relationship between such expression and the development of autoimmune disease remained to be elucidated prior to the present invention.

Programmed cell death or apoptosis is a fundamental mechanism in the development of the organism and occurs from mbryogenesis through ut life. Unfortunately, little is known about the process on the molecular level.

Of the two mechanisms thought to be important, the more classical type of programmed death is thought to require activation of a set of genes that lead to DNA fragmentation and subsequent apoptotic morphological changes. One gene thought to be involved is PD-1 which is specifically expressed after induction by a cell death signal. PD-1 is a member of the Ig supergene family that expresses a membrane receptor protein thought to associate with a tyrosine kinase.

A second membrane receptor-like protein, Fas, has also been reported to be involved in programmed cell death. A mutation in the *fas* gene has been implicated in the cause of the lymphoproliferative disorder seen in MRL-*lpr/lpr* autoimmune mice, however, the nature of the mutation has not yet been defined. Determining the nature of the *fas* defect which occurs in MRL-*lpr/lpr* mice would be a significant advance in that it would lead to an understanding of autoimmune disease such that effective treatment strategies could be designed.

An increased understanding of autoimmune processes will likely provide a significant insight into immune regulation in both health and disease and contribute to a broader understanding of the interaction of host and external factors in the immune system. Furthermore, elucidating the molecular basis of autoimmune diseases is of great importance as, without such knowledge, screening for effective therapeutic agents for use in treating many relatively common disorders, including rheumatoid arthritis, will continue to be severely limited. Additionally, without an indepth knowledge of the molecular mechanisms underlying autoimmunity, the rational design of therapeutics will remain virtually imp ssible.

SUMMARY OF THE INVENTION

By establishing the first link between endogenous retrovirus expression, defective apoptosis and autoimmune disease, the present invention seeks to overcome several drawbacks inherent in the art of autoimmune disease diagnosis and treatment. The discoveries disclosed herein concerning the inactivation of the *fas* apoptosis gene by an *ETn* retroviral insert have opened the way for the development of new molecular-based strategies for diagnosing and treating autoimmune and lymphoproliferative diseases.

This invention first provides novel nucleic acid segments and probes for use in molecular biological embodiments including diagnostic and screening assays. The inventors discovered that an *ETn* retroviral insert into the *fas* apoptosis gene is the molecular mechanism underlying the symptoms of an autoimmune mouse model. Accordingly, the nucleic acids of the present invention are generally purified nucleic acid segments, isolated free from total genomic DNA, which include a Fas cell surface protein-encoding sequence in combination with an *ETn* gene sequence, or the complements of such sequences.

In certain embodiments, nucleic acid segments are provided which will encode an apoptosis-defective Fas cell surface protein (also termed Fas antigen) with an *ETn* amino acid sequence insert, such as a Fas protein including an *ETn* sequence insert within an extracellular domain. In one exemplary embodiment, the *ETn* gene sequence will be inserted at position 232 of the *fas* gene, in another embodiment the coding sequence for the Fas protein may also include an additional triplet at position 240. Nucleic acid segments including an *ETn* insert may be exemplified by segments which have a sequence in accordance with the sequence of seq id no:3

or seq id no:4, however, the present invention is intended to encompass all *ETn* sequence insertions.

It will be understood that the nucleic acid segments may contain the entire *fas* gene, as described by Watanabe-Fukunaga *et al.* (1992a), or may contain smaller sections, as represented by, e.g., seq id no:7 and seq id no:8, so long as such *fas* sequences have *ETn* sequences inserted therein. The nucleic acid segments of the present invention may encode an apoptosis-defective Fas cell surface protein. Such a protein may include within its sequence an amino acid sequence in accordance with seq id no:2, and may be encoded by a DNA sequence which includes a sequence in accordance with seq id no:1, which may also include extended *fas* sequences so that the entire Fas antigen in encoded.

Alternatively, the nucleic acid segments of the present invention may be smaller so that they do not encode an entire protein. Purified nucleic acid segments with sequences in accordance with the nucleic acid sequences set forth in seq id no:1, seq id no:21 or seq id no:22, or the complements of such sequences, are contemplated to be particularly useful. Small nucleic acid segments which span the junction of the *fas* and *ETn* sequences, such as those represented by the nucleic acid sequences of seq id no:21 or seq id no:22, or sequences which comprise at least a ten nucleotide long stretch which corresponds to seq id no:21 or seq id no:22, are contemplated to be particularly useful.

As used herein, the term nucleic acid segment is intended to refer to DNA and RNA molecules which have been isolated free from total genomic or total cellular nucleic acids. Therefore, a nucleic acid segment of the present invention most often refers to a nucleic acid segment which is isolated away from total T cell nucleic

acids. Included within the term "nucleic acid segment" are segments which may be employed in the preparation of vectors, as well as the vectors themselves, including, for example, plasmids, cosmids, phage, viruses, and the like. It will be understood that the present invention also encompasses sequences which are complementary to the sequences listed herein, along with biological functional equivalents thereof, including naturally occurring variants and genetically engineered mutants.

The DNA segments and recombinant vectors of the present invention may variously include the DNA coding regions set forth herein, coding region bearing selected alterations or modifications in the basic coding region, or may encode larger polypeptides which nevertheless include disclosed sequences, particularly from seq id nos:1, 21 or 22. Nucleic acid molecules having stretches of 10 or 12 nucleotides or so, complementary to seq id nos:21 and 22 will have utility, for example, as hybridization probes. However, the total size of fragment, as well as the size of the complementary stretch(es), may be varied depending on the intended use or application of the particular nucleic acid segment. Nucleic acid fragments of up to 50 or 100 basepairs in length which include a sequence in accordance with, or complementary to, the sequences of seq id no:21 or seq id no:22 are particularly contemplated.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited

by the ease of preparation and use in the intended recombinant DNA protocol.

It will also be understood that this invention is not limited to the exact nucleic acid and amino acid sequences described herein. Therefore, DNA segments prepared in accordance with the present invention may also encode biologically functional equivalent proteins or peptides which have variant amino acid sequences. Such sequences may arise as a consequence of codon redundancy and functional equivalency which are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged.

The nucleic acid segments and probes of the present invention may be used in detecting apoptosis-defective T cells from animals or humans and may also be employed in molecular biological embodiments such as screening assays. However, they are not limited to such uses and also have utility in a variety of other embodiments, for example, as probes or primers in nucleic acid hybridization embodiments and in the expression of peptides and polypeptides for antibody generation.

The ability of these nucleic acid segments to specifically hybridize to *fas* and *ETn*-like sequences will enable them to be of use in various ways, e.g., as primers for the cloning of further portions of genomic DNA, and particularly, for the preparation of mutant species primers. The specific mutagenesis and subsequent analysis of various Fas proteins which caus apoptosis defects in T cells would be invaluable as a tool in more

precisely defining the apoptotic pathways in these cells and their interaction with disease processes. This would allow more effective drugs and therapeutic strategies to be designed to combat a variety of disorders.

Fas proteins (antigens) and Fas peptides and polypeptides, purified relative to their natural state, are also encompassed by the present invention. The invention particularly concerns Fas proteins and polypeptides which also include ETn amino acid sequences, as exemplified by an apoptosis-defective mutant Fas protein which includes within its sequence an amino acid sequence in accordance with seq id no:2. Fas proteins and peptides may contain only such sequences themselves or may be linked to other protein sequences, such as, e.g., 'natural' sequences derived from other T cell proteins or portions of 'engineered' proteins such as, e.g., glutathione-S-transferase (GST), ubiquitin, ß-galactosidase and the like. Antibodies having binding affinity for Fas/ETn combination proteins are also contemplated by the present inventors.

This invention also provides a method for identifying an apoptosis-defective T cell, which method generally comprises obtaining a DNA or RNA sample from a population of T cells suspected of containing apoptosis-defective T cells and probing said sample with a nucleic acid probe capable of differentiating between normal *fas* gene transcripts and aberrant *fas* gene transcripts. To "differentiate" in this manner generally requires the use of a nucleic acid probe, such as those described herein, which allows *fas* DNA or RNA from normal T cells to be identified and differentiated from *fas* DNA or RNA from apoptosis-defective T cells. This may be achi ved using criteria such as, e.g., number, pattern and size of *fas* nucleic acids, and more particularly, by the presence of an *Etn* sequence insert within the *fas* sequence.

The method for id ntifying apoptosis-defective T cells described herein may be employed as a method for, or part of a method for, identifying an individual at risk for developing systemic autoimmune disease. Therefore in addition to cells from animal models, and cultured cells, the T cells analyzed in such methods may obtained from an individual suspected of being at risk and for developing systemic autoimmune disease, or from a patient known to be presently or previously suffering from such a disorder. In such a method, the identification of apoptosis-defective T cells would be indicative of a disease state or the propensity to develop such a disease state.

To identify an apoptosis-defective T cell in accordance with the present invention one may employ Northern blotting technology, as described in the text and Figures 3 and 4 herein, and known to those of skill in the art (e.g., see Sambrook *et al.*, 1989). In this method, one would obtain RNA, and preferably, mRNA, from a population of T cells suspected of containing apoptosis-defective T cells, and probe the RNA with a nucleic acid probe capable of identifying normal *fas* gene transcripts and aberrant *fas* gene transcripts, preferably those including an *ETn* gene sequence insert, wherein a reduction in the amount of a normal *fas* gene transcript or the presence of an aberrant *fas* gene transcript is indicative of an apoptosis-defective T cell. Nucleic acid probes for use in such embodiments may include *fas* gene sequences, *ETn* gene sequences or both such sequences.

In certain embodiments concerning mouse cells, a reduction in the amount of *fas* gene transcripts estimated to be of about 2.2 kb in length, as compared to the l vel of such transcripts in normal mouse cells, is indicative of T cells which are apoptosis-defective. In *lpr/lpr*

mouse cells, multiple aberrant *fas* mRNA transcripts ranging from about 2 kb to about 9.5 and 10.5 kb were detected, with a prominent *ETn*-containing insert of 10.5 kb being particularly noticeable. Accordingly, an elevation in the levels of such transcripts is indicative of an apoptosis-defective T cell.

It is important to note that the size of normal and aberrant *fas* transcripts may vary between different species and cell lines, however, in light of the present invention, aberrant *fas* species will always be distinguishable from *fas* species in normal cells of the same species. It is therefore clear that using the technology described herein one may differentiate between normal and mutant *fas* transcripts, and thus identify apoptosis-defective T cells in an assay or screening protocol, regardless of the actual size and pattern of the aberrant transcripts themselves.

Another method which may be employed to identify an apoptosis-defective T cell and, therefore, to identify an individual at risk for developing systemic autoimmune disease, is based upon Southern blotting. In this method, the nucleic acids obtained for analysis would be DNA, and preferably, genomic DNA, which would be digested with one or more restriction enzymes and probed with a nucleic acid probe capable of hybridizing to normal-sized *fas* DNA bands and aberrant-sized *fas* DNA bands, preferably those which include an *ETn* gene sequence insert. A reduction in the amount of normal-sized *fas* DNA, or the presence of aberrant-sized *fas* DNA, e.g., *ETn*-including *fas* DNA, would be indicative of an apoptosis-defective T cell.

A large battery of restriction enzymes are commercially available and the conditions for Southern blotting are d scribed hereinbelow, suitable

modifications of which will be known to those of skill in the art (see e.g., Sambrook *et al.*, 1989, incorporated herein by reference). The utility of Southern blotting is exemplified herein in embodiments using mouse cells (see, e.g., Figure 1a) where an additional 5.3 kb of DNA within the extracellular domain of the genomic *fas* gene, as compared to normal mouse cells, was found to be indicative of T cells which are apoptosis-defective.

Kits for use in Southern and Northern blotting to identify apoptosis-defective T cells, or individuals at risk for developing systemic autoimmune disease, are also contemplated to fall within the scope of the present invention. Such kits will generally comprise a first container including *fas* or *ETn* nucleic acid probes, and preferably both; a second container including unrelated probes for use as controls; and optionally, a third container which includes one or more restriction enzymes.

In still further embodiments, the present invention concerns a method for identifying agents capable of promoting normal apoptosis in apoptosis-defective T cells, which agents are herein termed "candidate substances." It is contemplated that this screening technique will prove useful in the general identification of any compound that will serve the purpose of promoting or restoring the normal apoptotic mechanisms in such cells. As such, candidate substances which have activity in such assays would be good potential agents for use in the treatment of systemic autoimmune diseases. It will be understood that positive candidate substances, i.e., T cell apoptosis-promoting substances, and pharmaceutical compositions thereof, identified by the methods disclosed herein are encompassed by the present invention.

The screening methods of the invention generally include obtaining a composition containing apoptosis-

defective T cells, preferably those which express a Fas cell surface protein including an *ETn* gene sequence insert, and admixing or contacting this cell composition with a candidate substance. One would then determine the ability of the candidate substance to decrease the expression of aberrant *fas* gene transcripts, particularly those which include an *ETn* gene sequence insert; to decrease the expression of *ETn* gene transcripts; or to increase the expression of normal *fas* gene transcripts.

Any suitable method may generally be employed to identify normal and aberrant (*ETn* insert-including) *fas* gene transcripts and *ETn* gene transcripts. Preferred methods are those described hereinabove, particularly those utilizing *fas* and *ETn* probes in Northern blotting studies. The CD2-*fas* transgenic mouse studies presented herein demonstrate that *fas* transcription is less disrupted under conditions that suppress expression of the *ETn* retrotransposon. Therefore, in general, a candidate substance that produces a Northern blotting pattern positive for normal *fas* transcripts and negative for *ETn* transcripts (i.e., similar to that shown on the left hand side of Figure 4, as opposed to that shown on the right) would be indicative of a useful candidate substance.

Such candidate screening assays are relatively simple to set up and perform, and may be conducted in cell culture or by using an animal model such as the *lpr/lpr* mouse model which contains apoptosis-defective T cells. After contacting the cells with the candidate substance for an appropriate period of time, as may be achieved by administering the candidate substance to an animal, one would then perform an assay, preferably a Northern blot, to determine the levels of *fas* and *ETn* transcripts. A potentially useful substanc would promote a more normal Northern blot pattern as opposed to

a typical defective pattern, as exemplified by the data in Figure 4.

It is proposed that chemical compositions, man-made compounds and compounds isolated from natural sources, such as plants, animals or even sources such as marine, forest or soil samples, may be assayed for the presence of potentially useful candidate substances. However, it will be understood that the candidate substances to be screened could also be derived from or comprise known pharmaceutical agents, including cytokines, which are not currently used in conjunction with cell apoptosis or autoimmune diseases. The 'candidate substances' may be also be genes, oligonucleotides or anti-sense oligos. The suitability of the technique for use in such embodiments is exemplified by the CD2-*fas* transgenic mouse studies described herein.

In still further embodiments, the discoveries of the present invention are contemplated for use in designing new treatment strategies for autoimmune diseases. For example, drugs may be identified or designed to normalize transcription of genes important in tolerance induction and apoptosis, such as the *fas* gene and other apoptosis genes known to those of skill in the art, and to restore normal gene function despite the presence of a mutation. The inventors thus envision methods and compositions to modulate the expression of a retrovirus, either upward or downward depending on its mode of action and its association with tolerance related or lymphocyte activation genes, as part of the treatment for autoimmune diseases.

The invention thus concerns a method for treating systemic autoimmune disease comprising administering to a patient with such a disease an immunologically effective amount of a pharmaceutical composition capable of

promoting normal apoptosis in apoptosis-defective T cells. More specifically, this may be achieved by administering a pharmaceutical composition comprising a positive candidate substance identified by the screening assays of the invention, or by administering a composition comprising an anti-sense oligonucleotide specific for a nucleic acid segment which includes a Fas cell surface protein-encoding sequence in combination with an *ETn* gene sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Southern blot analysis of the *fas* gene in MRL-*lpr/lpr* and wild-type MRL-+/+ mice and 5′ Genomic map of Murine *Fas* Gene. Figure 1 consists of Figure 1a and Figure 1b.

Figure 1a. DNA was purified from thymuses of MRL-*lpr/lpr* (L) and wild-type MRL-+/+(+) mice. High molecular weight DNA was digested with the indicated restriction enzyme and probed with a HincII cDNA fragment corresponding to the extracellular domain fragment extending from 219-569 bp of the murine sequence.

Figure 1b. Restriction map derived by single and multiple double enzyme digestions, and probing with a cDNA fragment corresponding to either a 170 bp PstI-HincII cDNA fragment (+49 bp to +219 bp) which is entirely 5′ of the *ETn* insertion, a probe corresponding to the remainder of the extracellular domain which was a 345 bp HincII cDNA fragment (+219 bp to +569 bp), or the full-length *fas* cDNA probe (+49 to +1033 bp). The location of the 5.3 kb inserted DNA and the approximate location of Ex1, Ex2 and Ex3 was derived using the different probes on multiple identical blots. Additional

enzyme sites present in the 5.3 kb insert are indicated. R=EcoRI, B=BamHI, H=HindIII, P=PvuII.

Figure 2. An abnormal *fas* RNA containing *ETn* is expressed in MRL-*lpr/lpr* mice but not in wild type mice. Figure 2 consists of Figure 2a, Figure 2b and Figure 2c.

Figure 2a. The position of the *ETn* sequence inserted within the *fas* gene as determined by sequence analysis of cDNA prepared from the thymus of MRL-*lpr/lpr* and wild-type mice. The PCR primers used and their relative locations with the *fas* gene are indicated as P1, P2, P3 and P4. Also shown is the location of the 170 bp 5′ probe which is the PstI-HincII fragment, and the 345 bp extracellular domain probe which is the HincII fragment derived from the normal *fas* cDNA clone.

Figure 2b. PCR products using primers P2-P3 subjected to agarose gel electrophoresis and visualized by ultraviolet illumination in the presence of ethidium bromide. A unique larger PCR product was observed using thymic RNA from six different MRL-*lpr/lpr* mice.

Figure 2c. The insertion of the *ETn* sequence found within the otherwise normal extracellular coding region of the wild-type *fas* gene. The *fas* gene sequence (early portion, seq id no:7; late portion, seq id no:8) is numbered according to the numbering in the Watanabe-Fukunaga *et al.* (1992a), the position of which numbers are indicated as •. The nucleotide sequence of the *Etn*-derived insert (seq id nos:5 and 6) is numbered from position 1 of the insert, the position of which numbers are as indicated as ▾. The length of the inserted portion including the additional G residue (seq id no:4) is 168 bp. The *ETn* insert in the *fas* gene of MRL-*lpr/lpr* mice results in a novel sequence (seq id no:1) and an

in-frame amino acid sequence (seq id no:2) shown below the cDNA sequence.

Sequences in Figure 2c: The *Etn* nucleotide sequence corresponding to the nucleotide sequence of the insert from position 1 through position 45 (prior to the addition of a G at the corresponding position of the MRL-*lpr* sequence) is represented by seq id no:5. The *Etn* nucleotide sequence after the addition of a G at the corresponding position of the MRL-*lpr* sequence through position 168 is represented by seq id no:6. A continuous nucleotide sequence of 168 residues comprising the sequence of seq id no:5 linked directly to the sequence of seq id no:6 by a single additional nucleotide (A, C, T or G) is represented by seq id no:3. A continuous nucleotide sequence of 168 residues comprising the sequence of seq id no:5 linked directly to the sequence of seq id no:6 by a single additional G residue, as was found to occur in one particular instance, is represented by seq id no:4. The sections of the wild-type *fas* nucleotide sequence prior to, and after, the insert are represented by seq id no:7 and seq id no:8, respectively. The nucleotide sequence of the resultant MRL-*lpr* coding segment, which includes the *fas* gene segments and an *ETn*-derived insert, is represented by seq id no:1; and the corresponding amino acid sequence resulting from the altered MRL-*lpr* coding segment is represented by seq id no:2.

Figure 3. Northern blot analysis of *fas* RNAs from the thymus of wild-type MRL-+/+ and MRL-*lpr/lpr* mice. Figure 3 consists of Figure 3a, Figure 3b, Figure 3c and Figure 3d.

Thymus poly-A^+ RNA from the indicated mouse strains were analyzed by probing four identical blots with (a) a full l ngth *fas* cDNA probe, (b) a 5′ Pst-I/HincII *fas* cDNA probe corresponding to position 49-219, (c) a *fas*

cDNA probe corresponding to the 345 bp HincII fragment of extracellular domain, and (d) a 168 bp *ETn* probe derived from *ETn* sequences within the abnormal sized *fas* transcript obtained by PCR amplification of the extracellular domain of *fas* cDNA from *lpr/lpr* mice. MRL-+/+, BXSB male and NZB female mice were 2 mo of age. MRL-*lpr/lpr* mice were 1 mo old (lanes 4,6) and 3 mo old (lane 5). The upper arrows in panels a,b and c indicate the abnormal *fas* transcripts in MRL-*lpr/lpr* mice which correspond in size to a unique transcript which also hybridizes to the *ETn* probe used in panel d.

Figure 4. Decreased *ETn* expression in CD2-fas transgenic MRL-lpr/lpr mice. Poly-A RNA from thymus, lymph node (LN) and brain of 4 wk old CD2-*fas* transgenic and non-transgenic MRL-*lpr/lpr* mice was blotted as described in Figure 3. Figure 4 consists of Figure 4A, Figure 4B and Figure 4C.

Figure 4A. ETn expression is decreased in the thymus of 4 wk old CD2-fas transgenic MRL-lpr/lpr mice but not in age-matched non-transgenic littermate control mice.

Figure 4B. CD2-fas transgenic MRL-lpr/lpr mice have high levels of fas RNA in the thymus and LN but not in non-T cell sites including the brain. Non-transgenic litter mate control mice do not express fas.

Figure 4C. The blot was stripped and hybridized with a β-actin probe to ensure that nearly equal amounts of RNA was present in all samples.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The MRL-*lpr/lpr* mouse strain is a model of systemic autoimmune disease. The homozygous expression of the *lpr/lpr* gene leads to autoimmunity and lymphadenopathy in different strains of mice including MRL, C57BL/6, C3H, AKR, and Balb/c mice (Cohen & Eisenberg, 1991). In such mice, intrinsic defects of intrathymic T cell development exist, including defective deletion of self-reactive T cells (Zhou *et al.*, 1991; 1992) and expression of endogenous retroviruses (Kreig *et al.*, 1991). Expression of the 3′ long terminal repeat (ltr) of the endogenous mouse mammary tumor virus (MMTV) as a superantigen has been reported to influence the shaping of the repertoire of the normal immune response (Choi, *et al.*, 1991; Frankel *et al.*, 1991; Woodland *et al.*, 1991; Pullen *et al.*, 1992). However, the relationship between the expression of modified endogenous retroviruses in the thymus of autoimmune mice and the development of autoimmune disease was not defined prior the present invention.

A defect in Fas expression, a cell surface antigen which mediates apoptosis, has been described in MRL-*lpr/lpr* mice. A germline mutation in the *fas* gene which leads to abnormal Fas expression has been proposed to cause defective deletion of self-reactive T cells in the thymus of these mice (Itoh *et al.*, 1991; Watanabe-Fukunaga *et al.*, 1992b). One report identified a point mutation in the intracellular region of the *fas* gene in CBA/J-*lpr*^{cg} mice which is believed to be functionally significant (Watanabe-Fukunaga *et al.*, 1992b).

The *fas* gene also has been found to be abnormal in MRL-*lpr/lpr* mice in which Southern blot analysis indicated altered restriction enzyme digestion and *fas* RNA expression was not detectable in the thymus

(Watanabe-Fukunaga *et al.*, 1992b). These results led to the conclusion that the *fas* mutation in MRL-*lpr/lpr* mice was different from the mutation in CBA/J-*lpr*^{cg} mice, and that in MRL-*lpr/lpr* mice the mutation leads to disruption of normal transcription of the *fas* gene.

Different strains of *lpr/lpr* mice develop different types of lymphoproliferative autoimmune disease (Cohen & Eisenberg, 1991). Genetic differences between the different strains of *lpr/lpr* mice play a role in determining the levels in autoantibody production, the type and severity of autoimmune disease and extent of lymphoproliferation (Cohen & Eisenberg, 1991). Genes determining the severity of renal disease in mice expressing the *lpr/lpr* gene have been mapped to chromosome 7 and chromosome 12, whereas genes associated with arthritis, although known to exist, have not yet been mapped. The heterozygous expression of the *lpr* gene also leads to a less severe form of lymphoproliferative autoimmune disease. It is not yet known if these disease differences are related to differences in expression of the *fas* gene, or to the influence of other genes in the immune response.

It is apparent that, despite intensive efforts in the field, the molecular mechanisms underlying the Fas apoptosis defect in MRL-*lpr/lpr* mice had not been elucidated prior to the present invention. This lack of understanding thus prevented the development of the kind of useful screening assays which the art so clearly needs. The present inventors surprisingly found that the mutation of the *fas* gene is due to the insertion of a retroviral transposon of the *ETn* family (Shell *et al.*, 1987; 1990), resulting in inclusion of an *ETn* sequence within the coding region of the mature *fas* mRNA in the thymus.

Using different fragments of a *fas* cDNA probe, the inventors determined that the *lpr/lpr* mutation was a 5.3 kb insertion of DNA within the second intron of the *fas* gene. cDNA corresponding to this region was derived from thymic RNA from MRL-*lpr/lpr* and MRL-+/+ mice using the polymerase chain reaction. All thymic RNA samples from MRL-*lpr/lpr* mice yielded a unique product that was 168 bp larger than that of MRL-+/+ mice. Complete sequence analysis indicated that this inserted sequence had 98% homology with a sequence from the 3' LTR of the *ETn* transposon. RNA analysis indicated higher expression of *ETn* RNA in the thymus of MRL-*lpr/lpr* than MRL-+/+ mice. This mutation leads to abnormal transcription and splicing of the *fas* gene in MRL-*lpr/lpr* mice, resulting in 1/50th reduced amounts of normally spliced *fas* mRNA. The expression of *ETn* was found to be increased in the thymus of younger mice, but to decrease with age.

The inventors next analyzed the interdependence of *ETn* expression and abnormal *fas* expression in a CD2-*fas* transgenic mouse model in which a full length murine *fas* cDNA under the regulation of the CD2 promoter and enhancer was used to correct defective *fas* expression in T cells of MRL-*lpr/lpr* mice. In CD2-*fas* transgenic MRL-*lpr/lpr* mice, increased expression of *fas* mRNA results in decreased expression of *ETn* in the thymus. This indicates that the extent of interruption of the *fas* transcription by *ETn* is not constant, and that *fas* transcription is less disrupted under conditions that suppress expression of the *ETn* retrotransposon.

The results from these studies suggest that autoimmunity may result from the combination of the ability of predisposing background genes to facilitate the transcription of endogenous retroviruses, and the integration of a retrovirus or transposon n ar, or within, a gene of importance in tolerance induction. In

this type of genetic mechanism, endogenous retroviral sequences (ERS) act as autogenes to adversely modulate immune cell function creating a state of immunologic dysregulation. Herein, an autogene is defined as an ERS integrated into the mammalian chromosome in such a way that it promotes autoimmune disease, in a similar manner to oncogenes promoting cancer.

Since the predisposition to autoimmune disease dependent upon classical immune response genes is relatively weak, additional causative factors have long been suspected. In recent years, the role of infectious viruses has received much attention. Epstein-Barr, the agent of infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma in southeastern China, can function as a polyclonal B cell activator and it may be a co-factor in some human autoimmune diseases. Numerous autoimmune, arthritic, and dermatologic manifestations as well as a diffuse lymphoproliferative disorder can occur as a consequence of human immunodeficiency virus (HIV) infection. Some patients with systemic lupus erythematosus (SLE), Sjogren's Syndrome (SS) and scleroderma make antibodies which are reactive with the p24 *gag*-protein of HIV-1.

— The defective function of the *fas* protein in the MRL/*lpr* thymus, shown herein to be due to the insertion of a retroviral transposon of the *ETn* family, results in defective apoptosis. Failure or suppression of physiologic apoptosis is also important in cancer, where it is regulated by oncogenes. Cell death is a well-modulated and active process that may be blocked in some lymphomas and leukemias. *Bcl*-2 protein, an oncogene found in mitochondria, suppresses apoptosis in neurons as well as in lymphocytes. T lymphocytes that overexpress *bcl*-2 are resistant to apoptotic killing.

Certain types of oncogenes are mammalian genes which are captured by tumor viruses and reinserted into the host genome in a way that leads to carcinogenesis. Oncogenes and autogenes belong to the twin worlds of both virus and host. Just as the clinical consequence of oncogene activation is cancer, the clinical consequence of autogene activation is autoimmune disease. These pathologic effects may actually be quite similar since autoimmune disease is often accompanied by lymphoproliferation and may predispose to malignant lymphoma.

Autogene products may act as controlling elements regulating the immune response directly (e.g. as antigens) or indirectly (e.g. by controlling the lymphocyte growth cycle, particularly through apoptosis). Autogenes, like oncogenes, may not be a fixed genetic defect but rather inducible by endogenous triggers which increase the predisposition for development of disease. These triggers might be other viruses or genes, sex hormones, or neurotransmitters. As in the case of oncogenes, the genetic basis behind increased autogene expression may be a mutation or translocation leading to abnormal transcription or altered genetic coding regions, which in turn leads to altered function of the autogene. Possibly ERS evolved originally to augment the immune response, thereby imparting a selective advantage and leading gradually by natural selection to a human population better adapted to survive in a world full of infectious organisms but also more prone to autoimmune reactions. In any event certain ERS acquire the ability to become expressed in a way that promotes autoimmune disease.

Almost all endogenous retroviruses are non-infectious. Perhaps 5% of the mammalian genome arises through reverse transcriptase (RT) of retroviral

sequences. There are many mechanisms by which retroviruses can induce autoimmunity including polyclonal B cell activation, cytokine dysregulation, immune suppression, molecular mimicry, etc.

The ability of ERS to act as superantigens lends further evidence to their possible role as autogenes. Minor lymphocyte stimulating (Mls) antigens can act as endogenous superantigens that activate or induce the deletion of large portions of the T cell repertoire. They are encoded by mouse mammary tumor viruses (MMTV) that have integrated into the germ line as DNA proviruses. Hence, T cells bearing TCR Vβ-specific for the superantigen Mls-1a (encoded in the open reading frame of the 3' long terminal repeat of endogenous MMTV) can lead to deletion of T cells expressing Mls reactive Vβ regions. A murine leukemia virus (MuLV) with similar superantigen properties has been discovered. A rapid activation and proliferation of CD4' T cells is associated with the development of an immunodeficiency syndrome of mice caused by a replication-defective MuLV.

Retroviruses can also alter immune function by integration and disruption of either the structure or regulation of the host gene. Retrotransposons are retroviral sequences that are especially capable of this type of immune disfunction. Mtv retroviral sequences can also effect transcription of adjacent genes. Mtv-8 is an endogenous retrovirus located 4.6-kb upstream of a V*K* region gene (called V*K*9M) within the *K*-Ig locus. The proximity of these two genes results in reciprocal transcriptional activation. Mtv-8 transcription can be detected after juxtaposition of the *K*-enhancers to the normally silent provirus. Reciprocally, the frequency of V*K*9M rearrangement is 5- to 10-fold higher in spleens

from Mtv-8-positive mice compared to spleens from mice that lacked the Mtv-8 provirus.

ETn elements are 5.7-kb retrotransposons found in the murine genome. The *ETn* family of long-repeated sequences is abundantly transcribed in early mouse embryos from retroviral-like long terminal repeats (LTR). Nucleotide sequencing of two *ETn* elements did not reveal any long open reading frame or significant homology with other retroviral proteins. The genetic polymorphism, monitored by Southern blotting within and across mouse species, reflects a conserved mode of evolution for the *ETn* sequences.

The *ETn* retrotransposon has been identified in several autosomal recessive disease states in mice and man. The *ETn* transposon has inserted into the major mammalian-skeletal-muscle-chloride-channel (CIC-1) in myotonia mice, destroying its coding potential for several membrane-spanning domains (Steinmeyer *et al.*, 1991). In some cases, as in *fas*, the *ETn* is incorporated into the cDNA of genes that are mutated by this transposon. Genes induced by glucocorticoids in murine thymocytes and in the WEHI-7TG cell line contain sequences for the remnants of a mouse *ETn* (Baughman *et al.*, 1991).

The transposon is strongly expressed not only in embryonic cells but also in plasmacytomas, B lymphomas and T cell lines. *ETn* integration within a gene was first observed upstream of Vλ2 in the P3X63Ag8 cell line. A unique 2.2-kb mRNA is transcribed from Q6 and Q8 genes of the mouse MHC. The 3′ portion of Q8 contains extensive homology with the ETn transposon.

The ability of a retrovirus to disrupt the r gulation of host genes, as is the case for *ETn* in the

fas gene of the *lpr* mice, may be related to common control regions for expression of retroviruses and developmental genes in lymphocytes. Early development of B and T cells are also associated with a number of enhancer binding proteins which regulate T and B cell development. One transcription factor, *ets*, is found in the enhancer of TCRα, TCRβ, TCRδ, CD3Σ, CD3δ and IL2, and is also found in the enhancers of retroviruses including HIV-1, MuLV and *ETn*.

High levels of expression of ets during T cell development might also lead to increased transcription of *ETn* and disruption of *fas* transcription. This model was tested by the present inventors in a TCRβ transgenic MRL-*lpr/lpr* mouse. The TCRβ transgene results in a complete loss of lymphoproliferative disease and production of the unusual CD4 CD8 B220+ T cell in MRL-*lpr/lpr* mice. Examination of *ETn* expression in T cells of TCRβ transgenic *lpr/lpr* mice indicated that *fas* expression increased relative to non-transgenic *lpr/lpr* mice. Thus, the *ETn* mutation within *fas* is not a fixed mutation but is a site of active transcription which might disrupt normal transcription during early T cell development.

— The results obtained during the present studies give rise to the following conclusions. First, the entire complex of autoimmune and lymphoproliferative disease that occurs spontaneously in *lpr/lpr* mice is intimately linked to a defect in apoptosis. Second, the molecular basis of this defect is traceable to an *ETn* integrated into the *fas* apoptosis gene. Also, the inventors found that the major features of the disease do not develop when this aberrant *fas* gene is accompanied by a TCRβ transgene, suggesting that the apoptosis defect is compensated for, or otherwise bypassed by, the acti n of this normal TCR gene.

It is now clear that endogenous retroviruses, apoptosis and autogenes are linked to autoimmune diseases in a fundamental way which may be exploited in the development of new strategies to combat autoimmune diseases. For example, it is envisioned that the present invention may be utilized to screen for, or design, drugs to normalize autogene transcription which may restore normal gene function despite the presence of an autogene mutation. In addition, the invention may be employed to gain further information regarding autogene regulation which will allow a better definition of the precise triggers (both endogenous and exogenous) that cause abnormal autogene expression and disease. Since most cytokine and immunosuppressive drugs in current usage induce apoptosis in susceptible cells, it is contemplated that this invention will allow the development of drug therapy which will restore normal apoptosis in autoimmune patients.

The present invention has opened the way for new therapeutic strategies for autoimmune disease. Thus, modulation of the expression of a retrovirus, either upward or downward depending on its mode of action and its association with tolerance related or lymphocyte activation genes, is envisioned to be of use in inhibiting an autoimmune response even in the presence of an uncorrected genetic defect. More particularly, in the present case, the use of an agent which downregulates *ETn* expression and thus promotes normal *fas* gene expression is contemplated. Such 'agents' may be genetic constructs or pharmacological agents identified by the screening assays of the invention.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow

represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE I

CHARACTERIZATION OF THE MRL-*lpr/lpr fas* MUTATION

A. MATERIALS AND METHODS

1. Normal Mice. The original breeding pairs of MRL-*lpr/lpr* and MRL-+/+ mice used for all the studies described herein were obtained from the Jackson Laboratory (Bar Harbor, ME).

2. Southern Blot Analysis. MRL-*lpr/lpr* and MRL-+/+ mice were obtained from the Jackson Laboratory, Bar Harbor, ME. DNA was prepared from the thymus and digested with the indicated restriction enzymes. Approximately 10 μg of the digested DNA was separated on a 0.7% agarose gel, blotted to a nylon membrane and hybridized with ^{32}P-labelled cDNA probes.

3. Northern Blot Analysis. Five micrograms of poly $(A)^+$ RNA was denatured at 65°C for 5 min in electrophoresis buffer (0.4 M 3-morpholinopropanesulfonic acid, 0.1 M sodium acetate, 2 mM EDTA pH=7.0) containing 6% formaldehyde and 50% formamide, and size fractionated by electrophoresis through 1% agarose gels containing 0.6% formaldehyde. Gels were stained with ethidium bromide to assure integrity of the loaded RNA. RNA was transferred to nylon membranes (Nitroplus 2000, M.S.I.

Inc., Eastboro, MA) and baked for 2 h at 80°C in a vacuum oven. Membranes were prehybridized and then hybridized with 1 to 3 x 10^6 cpm/ml of different DNA probes that had been labeled with ^{32}P by random priming to a specific activity of 10^9 cpm/μg. Filters were then washed with 2x SSC + 0.1% SDS at 42°C for 30 min and then with 0.1 x SSC + 0.1% SDS at 60°C for 30 min; they were then exposed to Kodak XAR-2 film (Eastman Kodak, Rochester, NY) at -70°C with intensifying screens.

4. PCR Analysis. Thymuses of mice were homogenized and total RNA was extracted from the homogenates by the guanidinium-CsCl method. Total RNA (2-4 μg) from each tissue was used for cDNA synthesis followed by PCR amplification using the Perkin-Elmer RNA-PCR kit (Perkin-Elmer, Norwalk, CT). Reaction conditions were as specified by the manufacturer. An oligo (dT) primer was used to initiate cDNA synthesis. Thirty PCR cycles (1 min at 95°C; 1.5 min at 55 °C; 2.5 min at 72°C) were run followed by extension for 10 min, and the amplification products visualized after electrophoresis on agarose gels (1.0%) under ultraviolet illumination in the presence of ethidium bromide. Gels were blotted and hybridized to a labelled internal *fas* probe to verify that the bands were *fas* specific. The full length *fas* cDNA was obtained by PCR amplification of cDNA made from thymus mRNA from MRL-*lpr/lpr* or MRL-+/+ mice.

The full length cDNA PCR primers used were as described below (in all the following, the sequence positions are referenced to the published murine *fas* sequence of Watanabe-Fukunaga *et al*. (1992a)). PCR

primers P1 and P4, the sequences of which are shown below (seq id no:9 and seq id no:12, respectively), were employed to obtain the full length cDNA.

P1 = 5′-GGC-CGC-CCG-CTG-TTT-TCC-CTT-GCT-GCA-GAG-3′, position +20
P4 = 5′-ATT-GAC-ATT-GGC-AAC-TCC-TGG-TCT-3′, position 1110

Internal PCR primers P2 and P3, the sequences of which are shown below (seq id no:10 and seq id no:11, respectively), were used to obtain the extracellular domain cDNA which was sequenced.

P2 = 5′-CA-CAG-TTA-AGA-GTT-CAT-ACT-CAA-GGT-ACT-AAT-3′ position+93
P3 = 5′-AA-AGT-CCC-AGA-AAT-CGC-CTA-TGG-TTG-TTG-3′, position 540

Also used in the sequencing reactions were the universal vector 5′ primer, 5′-CTG-TGG-ATC-TGG-GCT-3′, position 53 (seq id no:13); and the following 5′ primers:

5′-TGT-CAA-CCA-TGC-CAA-CCT-3′, position 215
(seq id no:14)
5′-CGA-AAG-TAC-CGG-AAA-AGA-3′, position 608)
— (seq id no:15) -
5′-CGA-GAA-AAT-AAC-ATC-AAG-3′, position 773
(seq id no:16)

The 3′ universal vector primer, 5′-GAA-TCT-AGA-ACC-TCC-AGT-3′, position 656 (seq id no:17); and the following 3′ primers were also used in the sequencing reactions:

5′-TGT-GTT-CGC-TGC-GCC-TCG-3′, position 464
(seq id no:18)
5′-ACA-GAA-GGG-AAG-GAG-TAC-3′, position 293

(seq id no:19)
5'-GTT-GAG-GAC-TGC-AAA-ATG-3', position 245
(seq id no:20)

5. *Genomic Cloning*. High molecular weight DNA from the thymus of MRL-*lpr/lpr* and MRL-+/+ mice was digested with PstI and HincII. The digested sample was divided into several wells and electrophoresed through 0.8% agarose. After electrophoresis, a portion of the gel was blotted and probed with the 345 bp HincII fragment of *fas* and the 168 bp *ETn* fragment and another portion of the gel was sliced into thin sections representing different molecular weights. The gel slices correspond in size to bands hybridizing with the *fas* or *ETn* probes were extracted from the gel using Geneclean II (Bio 101, La Jolla, CA). DNA fragments ranging from 3.8 kb to 5.6 kb were amplified using the appropriate 3' or 5' *fas* cDNA primers in combination with *ETn* primers from the 168 bp sequence (Fig. 2) and published *ETn* sequences (Shell *et al.*, 1990). PCR products were cloned into the PCR 2000 vector (Invitrogen, San Diego, CA) for sequence analysis.

6. *Sequence Analysis*. Sequence analysis was carried out on double stranded DNA derived from PCR amplification and cloning into the PCR 2000 (Invitrogen, San Diego, CA). Sequence analysis was carried out in both directions using 5' and 3' universal primers and *fas* specific primers. For genomic sequencing, universal primers were used to determine the sequence of both the 3' end of the second exon and the 5' end of the third exon and flanking intronic sequences.

7. *DNA Probes*. The full-length murine *fas* cDNA probe (49 bp-1033 bp) was derived by PCR amplification of cDNA prepared from MRL-+/+ thymus mRNA as described above and using previously described *fas* prim rs (Watanabe-Fukunaga *et al.* (1992a). A probe corresponding to the first and

second exons of *fas*, which are 5′ of the normal *ETn* insertion, was the 170 bp PstI-HincII fragment (49 bp-219 bp) derived from the full length *fas* cDNA clone (Fig. 2a). A probe corresponding to the remainder of the extracellular domain was the 345 bp HincII fragment (219 bp-569 bp) derived from the full length *fas* cDNA clone. The *ETn* probe was the 168 bp *ETn* sequence that was isolated by PCR amplification of cDNA prepared from MRL-*lpr/lpr* thymus mRNA and reamplified using primers specific for the 5′ and 3′ sequences of *ETn*. The β-actin probe was a gift from Dr. K. Gordon (GenZyme Corp., Framingham, MA) (13) and may be produced as described by Roberts *et al*. (1992).

B. RESULTS

1. The *lpr* Mutation Results from a 5.3 kb Insertion of DNA

High molecular weight DNA from the kidney and thymus of MRL-*lpr/lpr* and MRL-+/+ mice was digested with various restricted enzymes and probed with a 345 base pair sequence corresponding to the extracellular domain of the *fas* cDNA (Fig. 1a). There was no difference in restriction fragment lengths between high molecular weight DNA from the kidney and the thymus. There was an additional 5.3 kb of DNA within the extracellular domain of the genomic *fas* gene from MRL-*lpr/lpr* mice as determined by restriction fragment length analysis of Southern blots prepared using multiple single and double enzyme digestions and hybridization with a 170 base pair cDNA probe corresponding to the first and second exons of *fas* cDNA (49 bp-219 bp), an extracellular domain probe (219 bp-569 bp), and a full length fas cDNA probe (49 bp-1033 bp).

Th inserted DNA contained additional digestion sites for EcoRI, HindIII and PvuII but not for BamH1 (Fig. 1b). Using probes corresponding to exon 1 and exon 2, or a HincII cDNA fragment corresponding to the extracellular domain, the insert was localized to the region of the *fas* gene corresponding to the second intron (Fig. 1b).

2. Insertion of ETn in the Extracellular Domain of *fas*

To determine if the *lpr/lpr* mutation in the extracellular domain of the *fas* gene results in abnormal *fas* RNA, cDNA corresponding to the extracellular domain was derived from thymus RNA from several MRL-*lpr/lpr* and MRL-+/+ mice using the polymerase chain reaction and primers P2-P3 (Fig. 2a). All RNA samples from the thymus of different MRL-*lpr/lpr* mice yielded a unique polymerase chain reaction product that was 168 bp larger than that of wild-type MRL-+/+ mice (Fig. 2b).

The mutation of the *fas* gene in MRL-*lpr/lpr* mice was confirmed to be in the extracellular domain by sequencing, using full length primers P1-P4 (Figure 2a) and using the extracellular *fas* cDNA clones from MRL-*lpr/lpr* and MRL-+/+ mice. The sequence of the transmembrane and cytoplasmic domains were identical in MRL-*lpr/lpr* and MRL-+/+ mice. Complete sequence analysis of cDNA corresponding to the extracellular domain of the *fas* gene was carried out using two different MRL-*lpr/lpr* mice and indicated that there was a 168 bp insert into the *fas* cDNA sequence at position 232 of an otherwise normally encoded extracellular domain (Fig. 2c).

This 168 bp sequence was analyz d by a GenBank search and found to exhibit an extremely high degree of homology with a s quenc from the 3' LTR of the *ETn* retroviral transposon (portions of which ar represented

by seq id no:5 and seq id no:6, Figure 2c). The inserted sequence within the *fas* cDNA is 98%-99% homologous to portions of *ETn* sequences previously found to be integrated into the Ig locus of mice, namely the sequences MUS *ETn* Xi (bp 1120-1285; Shell *et al.*, 1987;1990) and Mus *ETn* IgM (bp 270-435; Weiss & Johansson, 1989).

The differences between the previously identified *ETn* sequences and that found within the *fas* gene are the exchange of A for C at *ETn* position 22, the exchange of G for T at *ETn* position 66, and the insertion of an additional G residue at position 43 of the MRL-*lpr/lpr* *ETn* combined sequence. The nucleotide sequence of the MRL-*lpr* coding segment which has the *ETn*-derived insert in the *fas* gene is represented by seq id no:1, and the corresponding amino acid encoded by this altered, or mutated, MRL-*lpr* sequence is represented by seq id no:2 (Figure 2C). One MRL-*lpr/lpr* mice was found to have an additional AAA triplet at position 240 of the normal murine *fas* cDNA (Watanabe-Fukunaga *et al.* (1992a), making a total of four AAA codons in this region of the sequence.

3. **Aberrant *fas* Gene Expression**

A full length *fas* cDNA was used to probe northern blots of poly-A RNA prepared from the thymus of MRL-+/+, MRL-*lpr/lpr*, MRL-*lpr*/+, BXSB male and NZB mice and from the BW5147 cell line. In MRL-+/+ mice there was a 2.2 kb normal sized *fas* cDNA (Fig. 3a, lane 1-3). In contrast, in 1 mo old MRL-*lpr/lpr* mice there were multiple bands ranging from 2 kb to 10.5 kb (Fig. 3a, lane 4,6). Fas expression was highest in the thymus of 1 mo old MRL-*lpr/lpr* mice, and decreased in 3 mo old mice (Fig. 3a, lane 5).

When identical blots were hybridized with a 170 bp PstI/HincII *fas* cDNA fragment corresponding to the first and second exons, *fas* expression in MRL-*lpr/lpr* mice was very low compared to *fas* expression in MRL-+/+ mice (Fig. 3b, lane 4-6). A faint abnormal high molecular weight species of 10.5 kb was present using this 5′ probe (Fig. 3b, lane 4, arrow). When blots were probed with a 345 bp HincII *fas* cDNA fragment corresponding to the extracellular domain of *fas*, the primary species of RNA expressed in the thymus of young MRL-*lpr/lpr* mice was a high molecular weight 10.5 kb and 9.5 kb transcript (Fig. 3c).

These results indicate that the *fas* mutation leads to production of abnormal high molecular weight *fas* transcripts in the thymus. There was high expression of the 2.2 kb *fas* transcript in (MRL-*lpr/lpr* x MRL-+/+)F_1 mice, and also in BXSB male and NZB autoimmune mice (Fig. 3a,b,c; lanes 7-9). Expression of normal levels of *Fas* RNA in BXSB and NZB mice indicates that autoimmune disease in these mice is not related to defective expression RNA.

The 168 bp *ETn* probe, derived from within the *fas* cDNA prepared from thymus RNA of MRL-*lpr/lpr* mice, strongly hybridized to a 5.7 kb full-length *ETn* transcript which was expressed in the thymus of younger MRL-*lpr/lpr* mice (Fig. 3d, lane 4,6), but not strongly expressed in the thymus of older MRL-*lpr/lpr* mice (Fig. 3d, lane 5) or in the thymus of MRL-+/+ mice (Fig. 3d, lanes 1-3). RNA corresponding to the full-length 5.7 kb *ETn* transcripts was also abundant in the thymus of MRL-*lpr/+* and BXSB mice, and also in the BW5147 cell line. The largest *fas* transcript corresponds in size to an abnormal 10.5 kb *ETn* transcript in MRL-*lpr/lpr* mice (arrows, lanes 4; Fig. 3a,b,c,d) suggesting the presence of the *ETn* sequence within one of the abnormal sized high

molecular weight *fas* transcripts of MRL-*lpr/lpr* mice. A higher approximately 10.5 kb molecular weight *fas* transcript was also present in the thymus of MRL-*lpr/+* and BXSB mice, but not NZB mice or the BW5147 cell line (Fig. 3*a*, lanes 7-10).

4. Germline Organization of the Mutated *fas* Gene in lpr Mice

Using PCR primer pairs to the 3′ end of *fas* exon 2 and the 5′ end of the 168 bp *ETn* found in the *fas* transcript or the 5′ end of *fas* exon 3 and the 3′ end of *ETn*, the sequence of cloned genomic fragments isolated using the 345 bp HincII fragment of *fas* and the 168 bp *ETn* fragment were determined. In MRL-*lpr/lpr* mice the *ETn* sequence began at the 5′ terminal of exon 2 and continued for an additional 5.3 kb. There was a conserved splice consensus nucleotide sequence on the 3′ end of exon 2-intron 2 (G/A) and on the 5′ end of the 168 bp *ETn* (A/G) which was found to be spliced into the *fas* transcript. Also, there was a conserved splice consensus at the 3′ end of the 168 bp *ETn* (A/G) and on the 5′ end of the *fas* exon 3 (A/G). These splice consensus sequences allow for splicing of the 168 bp *ETn* into the *fas* cDNA. Additional *ETn* sequence was present in the MRL=*lpr/lpr* mice directly adjacent to the 3′ terminus of the second exon. In MRL-+/+ and MRL-*lpr/lpr* mice there was an additional 3.5 kb of intron sequence consistent with the restriction map (Fig. 1).

EXAMPLE II

DOWNREGULATION OF ETn IN CD2-fas TRANSGENIC MRL-lpr/lpr MICE

A. METHODS

1. Production of CD2-fas Transgenic MRL-lpr/lpr Mice. The 1.1 kb full length *fas* cDNA was obtained by PCR amplification of cDNA made from thymus mRNA from MRL-+/+ mice as described herein. This was cloned into an EcoRI site in front of exon 1 of a human CD2 minigene consisting of 5.5 kb of 5′ flanking sequence, exon 1, the first intron, fused exons 2 to 5 and 2.1 kb of the 3′ flanking sequence. The 3′ sequence has been shown to be sufficient to allow T cell specific, copy-dependent, integration-independent expression in transgenic mice (Lang *et al.*, 1988; Greaves *et al.*, 1989).

MRL-*lpr/lpr* male and female mice were obtained from the Jackson Laboratory, Bar Harbor, ME. Single cell MRL-*lpr/lpr* embryos were produced, injected with approximately 100 copies of the CD2-*fas* transgene, and then placed into the distal oviduct of CD1 pseudopregnant female mice. Tail DNA prepared from offspring was digested with EcoRI and probed with a ^{32}P labeled full length *fas* cDNA to identify CD2-*fas* transgenic mice.

B. RESULTS

To determine if high *ETn* expression was dependent on abnormal *fas* expression, CD2-*fas* transgenic mice were produced that utilized a full-length murine *fas* cDNA under the regulation of the CD2 promoter and enhancer (8-9) to correct defective *fas* expression in T cells of MRL-*lpr/lpr* mice. The presence of the *fas* transgene resulted in reduction of expression of *ETn* in th thymus,

suggesting that high *ETn* expression is related to abnormal *fas* expression (Fig. 4a). Northern blot analysis indicated that there was high expression of the *fas* transgene in the thymus and lymph node, but not in the brain of 4 wk old CD2-*fas* transgenic MRL-*lpr/lpr* mice (Fig. 4b).

C. DISCUSSION

ETn retrotransposon transcription occurs during early embryonic development in mice (Wang *et al.*, 1992). Increased expression of endogenous retroviruses in the thymus of autoimmune strains has been proposed to be related to development of autoimmune disease (Gourley *et al.*, 1992). Inhibition of translation of retroviral transcripts by antisense RNA has been reported to result in increased proliferation of lymphocytes leading to the speculation that full-length retroviral transcripts and protein products are a compensatory mechanism for increased lymphocyte proliferation in autoimmune mice (Krieg *et al.*, 1989). The present data suggests a second mechanism of association of retroviruses with autoimmunity, in that increased retroviral expression is be related to defective *fas* expression in MRL-*lpr/lpr* mice.

The *ETn* retrotransposon in the second intron of the *fas* gene might interfere with *fas* expression by promoting abnormal transcription initiation and interfering with abnormal splicing. High expression of *ETn* correlates with high expression of an abnormal large sized *fas* transcript with a molecular weight of approximately 10.5 kb (Fig. 3; lane 4,6). The largest *fas* transcript corresponds in size with an abnormal *ETn* transcript of the same size. This transcript contains both 5′ and 3′ *fas* cDNA sequences because it is detected by both the 5′ 170 bp Pst-1/HincII *fas* probe, and the 3′ 345 bp HincII

fas probe. A lower unusual *fas* transcript with an approximate molecular weight of 7.5 kb which retains 5' and 3' *fas* sequences (Fig. 3, lane 4) does not hybridize with the 168 bp *ETn* probe indicating that the 168 bp portion of *ETn* is spliced out of this transcript. Other aberrant splicing events can lead to *fas* transcripts which contain only 168 bp of *ETn* sequences (Fig. 2). These results suggest that thymic developmental factors which lead to high *ETn* expression also promote production of an abnormally large *fas* transcript in MRL-*lpr/lpr* mice due to the integration of *ETn* within the second intron of the *fas* gene.

In MRL-*lpr*/+ heterozygous mice, the inventors found an increased expression of *ETn* and abnormal *fas*, despite the presence of apparently normal levels of *fas* transcription from the unmutated allele. It is possible that abnormal *fas* and high *ETn* are expressed in a subpopulation of thymocytes that express low levels of normal *fas* and exhibit abnormal thymic development. This was investigated in CD2-*fas* transgenic MRL-*lpr/lpr* mice. In these mice, *fas* expression is regulated by the CD2 promoter/enhancer which results in high expression of *fas* in all thymocytes, and elimination of *ETn* expression (Fig. 4). These results suggest that *fas* expression and *ETn* expression are functionally related.

The inventors also observed that *ETn* expression is decreased and *fas* expression is partially normalized in TCRβ transgenic mice. They previously demonstrated that in TCRβ transgenic mice, there is nearly total elimination of the $CD4^-CD8^-B220^+$ subpopulation of T cells and lymphoproliferation, but not elimination of autoimmunity (Mountz *et al.*, 1990; Zhou *et al.*, 1993). The inventors recently demonstrated that there is decreased apoptosis of thymocytes of MRL-*lpr/lpr* mice and an increase of a large, proliferating $CD4^+CD8^+$

subpopulation of thymocytes. The TCRβ transgene was found to reduce these large, proliferating $CD4^+CD8^+$ thymocytes and there was no difference between this population in TCRβ transgenic MRL-*lpr/lpr* mice the same population in MRL-+/+ mice. These results suggested that the presence of the TCRβ transgene corrected the defect in early T cell development related to lymphoproliferation despite the presence of a germline mutation of the *fas* apoptosis gene.

Rearrangement of the TCRβ chain gene has been proposed to play a critical role in early T cell development in the thymus (Teh *et al.*, 1992). The TCRβ transgene suppresses rearrangement of the endogenous TCRβ gene (Uematsu *et al.*, 1988). Suppression of rearrangement of the endogenous TCRβ gene might accelerate T cell maturation resulting in decreased levels of retroviral LTR and eukaryotic gene enhancer binding proteins associated with T cell development (Thompson *et al.*, 1992). Prevention of aberrant transcription initiation at the site of the *ETn* integration within the second intron of the murine *fas* gene could result in normal transcription initiation from the 5′ end of the *fas* gene. This would lead to the observed increased levels of *fas* expression in the thymus of the TCRβ transgenic MRL-*lpr/lpr* mice. This interpretation is consistent with the concept that *ETn* expression and abnormal *fas* expression are functionally related in *lpr* mice.

Abnormal *fas* expression and T cell development in the thymus of autoimmune mice might lead to continued high expression of retroviruses. Abnormal populations of T cells, or B cells found in the periphery of autoimmune mice exhibit common features of developmental defects and retrovirus expression. In the case of the *lpr/lpr* gene the present data suggest that retrovirus expression is

intimately related to abnormal *fas* gene transcription and abnormal lymphocyte development.

EXAMPLE III

Fas EXPRESSION IN DIFFERENT T CELL SUBSETS

LN T cells from MRL-*lpr/lpr* and MRL-+/+ mice were sorted (50,000 cells/sample) by flow cytometry into either normal $CD4^+CD8^-$ or $CD4^-CD8^+$ T cells, abnormal $Thy1^+B220^+$ T cells, or $Thy1^-B220^+$ cells. Quantitative PCR analysis indicated that there was no *Fas* RNA expression by the $Thy1^+B220^+$ subpopulation of LN T cells from MRL-*lpr/lpr* mice. In contrast, nearly normal levels of *Fas* RNA was expressed on the $CD4^+$ or $CD8^+B220^-$ T cells.

Quantitative PCR analysis of FACS sorted thymocytes indicated that *Fas* RNA expression is highly regulated during normal thymocytes development. *Fas* RNA was not expressed on normal immature $CD4^-CD8^-$ or large proliferating $CD4^+CD8^+$ thymocytes, but was present on small, non-proliferating $CD4^+CD8^+$ thymocytes. However, *Fas* expression was not evident in mature single positive $CD4^+CD8^-$ or $CD4^-CD8^+$ thymocytes. Absence of *Fas* on mature thymocytes might be due to down-regulation of *Fas* expression, or due to apoptosis of thymocytes that express *Fas*. In the thymus MRL-*lpr/lpr* mice, *Fas* expression was abnormally expressed on the large proliferating $CD4^+CD8^+$ population as well as in the mature $CF4^+$ or $CD8^+$ (single positive) population.

* * *

While the compositions and m thods of this invention have been described in terms of preferred embodiments, it

will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The references listed below are incorporated herein to the extent that they supplement, explain, provide a background for or teach methodology, techniques and/or compositions employed herein.

Baughman, G., Harrigan, M.T., Campbell, N.F., Nurrish, S.J. and Bourgeois, S., (1991) *Mol. Endocrinol.*, 5, 637-644

Choi, Y., J.W. Kappler and P. Marrack, *Nature* 350,203 (1991)

Cohen, P.L. and R.A. Eisenberg, *Ann. Rev. Immunol.* 9, 243 (1991).

Frankel, W.N., C. Rudy, J.M. Coffin and B.T. Huber, *Nature* 349,526 (1991)

Gourley, M.F., W.J. Kisch, C.F. Mojcik, L.B. King, A.M. Krieg, and A.D. Steinberg. 1992. *Cell Biol.* 11:253.

Greaves, D.R., F.D. Wilson, G. Lang, and D. Kioussis. 1989. *Cell* 56:979.

Harris, E. D., Jr. *N. Eng. J. Med.* 322, 1277-89 (1990)

Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata, *Cell* 66,233 (1991).

Krieg, A.M., W.C. Gause, M.F. Gourley and A.D. Steinberg, *J. Immunol.* 143, 2448 (1989).

Krieg, A.M., M.F. Gourley and A.D. Steinberg, *J. Immunol.* 146, 3002 (1991).

Lang, G., D. Wotton, M.J. Owen, W.A. Sewell, M.H. Brown, D.Y. Mason, M.J. Crumpton, and D. Kioussis. 1988. *EMBO J.* 7:1675.

Lawrence, R. C., Hochberg, M. C., Kelsey, J. L., McDuffie, F. C., Medsger, T. A. Jr., Felts, W. R., and Shulman, L. E. *J. Rheumatol.* 16, 427-441 (1989).

Mountz, J.D., T. Zhou, J. Eldridge, K. Berry, and H. Blüthmann. 1990. *J. Exp. Med.* 172:1805.

Okamoto *et al. J. Exp. Med.* 175, 71 (1992).

Pullen, A.M., Y. Choi, E. Kushnir, B. Kappler and P. Marrack, *J. Exp. Med.* 175, 41 (1992).

Roberts, B., P. DiTullio, J. Vitale, K. Hehir, and K. Gordon. 1992. *Gene* 121:255.

Shell, B., P. Szurek and W. Dunnick, *Mol. Cell. Biol.* 7, 1364 (1987)

Shell, B.E., J.T. Collins, L.A. Elenich, P.F. Szurek and W.A. Dunnick, *Gene* 86, 269 (1990).

Steinmeyer, K., Klocke, R., Ortland, C., *et al.*, (1991) *Nature*, 354, 304-308

Teh, H.S., H. Kishi, B. Scott, P. Borgulya, H. von Boehmer, and P. Kisielow. 1992. *Dev. Immunol.* 1:1.

Thompson, C.B., C.Y. Wang, I.C. Ho, P.R. Bohjanen, B. Petryniak, C.H. June, S. Miesfeldt, L. Zhang, G.J.

Nabel, and B. Karpinski. 1992. *Mol. Cell. Biol.* 12:1043.

Uematsu, Y., S. Ryser, Z. Dembic, P. Borgulya, P. Krimpenfort, A. Berns, H. von Boehmer, and H. Steinmetz. 1988. *Cell* 52:831.

Wang, C.Y., B. Petryniak, I.C. Ho, C.B. Thompson, and J.M. Deiden. 1992. *J. Exp. Med.* 175:1391.

Watanabe-Fukunaga, R., C.I. Brannan, N., Itoh, S. Yonehara, N.G. Copeland, N.A. Jenkins, and S. Nagata, *J. Immunol.* 148, 1274 (1992a).

Watanabe-Fukunaga, R., C.I. Brannan, A.N.G. Copeland, N.A. Jenkiens and S. Nagata, *Nature* 356, 314 (1992b).

Weiss, S., and B. Johansson, *J. Immunol.* 143, 2384 (1989).

Woodland, D.L., M.P. Happ, K.J. Gollob and E. Palmer, *Nature* 349, 529 (1991)

Zhou, T., H. Blüthmann, J. Eldridge, K. Berry and J.D. Mountz, *J. Immunol.* 147, 466 (1991)

Zhou, T., H. Blüthmann, J. Zhang, C.K. Edwards and J.D. Mountz, *J. Exp. Med.* 176, 1063-1072 (1992) In Press.

Zhou, T., H. Blüthmann, J. Eldridge, K. Berry, and J.D. Mountz. 1993. Origin of $CD4^-CD8^-B220^+$ T cells in MRL-*lpr/lpr* mice: clues from a TCR β mouse. *J. Immunol.* 150, 3651-3667 (in press).

SEQUENCE LISTING

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(B) COMPUTER: IBM PC COMPATIBLE

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: WORDPERFECT 5.1

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(B) FILING DATE: FILED CONCURRENTLY

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(B) TELEFAX: 713-789-2679

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 216 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGCTGTCAAC CATGCCAACC TGCTCGAGCG GCCTTCTCAG TCGAACCGTT CACGTTGCGA 60

GCTGCTGGCG GCCGCAACAT TTTGGCGCCT GAACAGGGAC CTGAAGAATG GCAGAGAGAT 120

GCTAAGAGGA ACGCTGCATT GGAGCTCCAC AGGAAAGGAT CTTCGTATCG GACATCGGAG 180

CAACGGACAG GTAAAAAAAA AGTTGAGGAC TGCAAA 216

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 72 amino acid residues
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Cys Cys Gln Pro Cys Gln Pro Ala Arg Ala Ala Phe Ser Val Glu Pro
 1               5                  10                  15

Phe Thr Leu Arg Ala Ala Gly Gly Arg Asn Ile Leu Ala Pro Glu Gln
            20                  25                  30

Gly Pro Glu Glu Trp Gln Arg Asp Ala Lys Arg Asn Ala Ala Leu Glu
        35                  40                  45

Leu His Arg Lys Gly Ser Ser Tyr Arg Thr Ser Glu Gln Arg Thr Gly
    50                  55                  60
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Lys Lys Lys Val Glu Asp Cys Lys
65 70

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 168 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCTCGAGCGG CCTTCTCAGT CGAACCGTTC ACGTTGCGAG CTGCTXGCGG CCGCAACATT 60

TTGGCGCCGG AACAGGGACC TGAAGAATGG CAGAGAGATG CTAAGAGGAA CGCTGCATTG 120

GAGCTCCACA GGAAAGGATC TTCGTATCGG ACATCGGAGC AACGGACA 168

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 168 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCTCGAGCGG CCTTCTCAGT CGAACCGTTC ACGTTGCGAG CTGCTGGCGG CCGCAACATT 60

TTGGCGCCGG AACAGGGACC TGAAGAATGG CAGAGAGATG CTAAGAGGAA CGCTGCATTG 120

GAGCTCCACA GGAAAGGATC TTCGTATCGG ACATCGGAGC AACGGACA 168

(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTCGAGCGG CCTTCTCAGT CGAACCGTTC ACGTTGCGAG CTGCT 45

(7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 122 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGGCCGCAA CATTTTGGCG CCGGAACAGG GACCTGAAGA ATGGCAGAGA GATGCTAAGA 60

GGAACGCTGC ATTGGAGCTC CACAGGAAAG GATCTTCGTA TCGGACATCG GAGCAACGGA 120

CA 122

(8) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGCTGTCAAC CATGCCAACC T 21

(9) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGTAAAAAAA AAGTTGAGGA CTGCAAA 27

(10) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGCCGCCCGC TGTTTTCCCT TGCTGCAGAG 30

(11) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CACAGTTAAG AGTTCATACT CAAGGTACTA AT 32

(12) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AAAGTCCCAG AAATCGCCTA TGGTTGTTG 29

(13) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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(14) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

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(15) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGTCAACCAT GCCAACCT 18

(16) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGAAAGTACC GGAAAAGA 18

(17) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGAGAAAATA ACATCAAG 18

(18) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAATCTAGAA CCTCCAGT 18

(19) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGTGTTCGCT GCGCCTCG 18

(20) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ACAGAAGGGA AGGAGTAC 18

(21) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTTGAGGACT GCAAAATG 18

(22) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAACCTGCTC GA 12

(23) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CGGACAGGTA AA 12

CLAIMS

1. A purified nucleic acid segment, isolated free from total genomic DNA, which includes a Fas cell surface protein-encoding sequence in combination with an *ETn* gene sequence.

2. The nucleic acid segment of claim 1, further defined as encoding an apoptosis-defective Fas cell surface protein wherein the *ETn* gene sequence is inserted within the coding sequence for the Fas protein.

3. The nucleic acid segment of claim 2, wherein the *ETn* gene sequence is inserted at position 232 of the *fas* gene.

4. The nucleic acid segment of claim 3, wherein the *ETn* gene sequence insert includes within its sequence a DNA sequence in accordance with seq id no:3.

5. The nucleic acid segment of claim 4, wherein the *ETn* gene sequence insert includes within its sequence a DNA sequence in accordance with seq id no:4.

6. The nucleic acid segment of claim 5, wherein the *ETn* gene sequence insert has the DNA sequence of seq id no:4.

7. The nucleic acid segment of claim 2, wherein the apoptosis-defective Fas cell surface protein includes

within its sequence an amino acid sequence in accordance with seq id no:2.

8. The nucleic acid segment of claim 7, wherein the coding sequence for the Fas protein includes within its sequence a DNA sequence in accordance with seq id no:1.

9. The nucleic acid segment of claim 2, wherein the coding sequence for the Fas protein includes an additional triplet at position 240.

10. The nucleic acid segment of claim 1, further defined as comprising at least a ten nucleotide long stretch which corresponds to the nucleic acid sequence of seq id no:21 or seq id no:22.

11. The nucleic acid segment of claim 10, further defined as comprising at least a twelve nucleotide long stretch which corresponds to the nucleic acid sequence of seq id no:21 or seq id no:22.

12. The nucleic acid segment of claim 10, further defined as comprising a nucleic acid fragment of up to 100 basepairs in length.

13. The nucleic acid segment of claim 12, further defined as comprising a nucleic acid fragment of up to 50 basepairs in length.

14. A purified nucleic acid segment with a sequence in accordance with the nucleic acid sequence of seq id no:1, seq id no:21 or seq id no:22 or the complement of such a sequence.

15. An apoptosis-defective mutant Fas protein which includes within its sequence an amino acid sequence in accordance with seq id no:2.

16. A method of identifying an apoptosis-defective T cell, comprising:

obtaining mRNA from a population of T cells suspected of containing apoptosis-defective T cells; and

probing said mRNA with a nucleic acid probe capable of identifying *fas* gene transcripts or *ETn* gene transcripts, wherein a reduction in the amount of a normal *fas* gene transcript or the presence of an aberrant *fas* gene transcript including an *ETn* gene sequence is indicative of an apoptosis-defective T cell.

17. The method of claim 16, wherein the nucleic acid probe includes a *fas* gene sequence.

18. The method of claim 16, wherein the nucleic acid probe includes an *ETn* gene sequence.

19. The method of claim 16, wherein the nucleic acid probe includes a *fas* gene sequence in combination with an *ETn* gene sequence.

20. The method of claim 16, wherein the population of T cells is obtained from an individual suspected of being at risk for developing systemic autoimmune disease and wherein the identification of apoptosis-defective T cells is a positive indication of such a risk.

21. A method of identifying an apoptosis-defective T cell, comprising:

obtaining genomic DNA from a population of T cells suspected of containing apoptosis-defective T cells;

digesting said DNA with one or more restriction enzymes; and

probing said digested DNA with a nucleic acid probe capable of hybridizing to normal-sized *fas* DNA bands and aberrant-sized *fas* DNA bands which include an *ETn* gene sequence insert, wherein a reduction in the amount of normal-sized *fas* DNA bands or the presence of aberrant-sized *fas* DNA bands including an *ETn* gene sequence is indicative of an apoptosis-defective T cell.

22. A method for identifying a candidate substance capable of promoting normal apoptosis in apoptosis-defective T cells, comprising:

containing apoptosis-defective T cells which express a Fas cell surface protein including an *ETn* gene sequence insert with a candidate substance; and

determining the ability of the candidate substance to decrease the expression of aberrant *fas* gene transcripts which include an *ETn* gene sequence insert, to decrease the expression of *ETn* gene transcripts or to increase the expression of normal *fas* gene transcripts.

23. The method of claim 22, wherein the apoptosis-defective T cells are located within an experimental animal and the candidate substance is administered to the animal.

24. A T cell apoptosis-promoting substance identified by the method of claim 22.

25. A pharmaceutical composition for use in treating systemic autoimmune disease comprising a substance identified by the method of claim 22 dispersed in a pharmacologically acceptable vehicle.

26. A method for treating systemic autoimmune disease comprising administering to a patient with autoimmune disease an effective amount of a composition capable of promoting normal apoptosis in apoptosis-defective T cells.

27. The method of claim 26, wherein said composition comprises an anti-sense oligonucleotide specific for a nucleic acid segment which includes a Fas cell surface protein-encoding sequence in combination with an *ETn* gene sequence.

28. The method of claim 26, wherein said composition is a composition in accordance with claim 25.

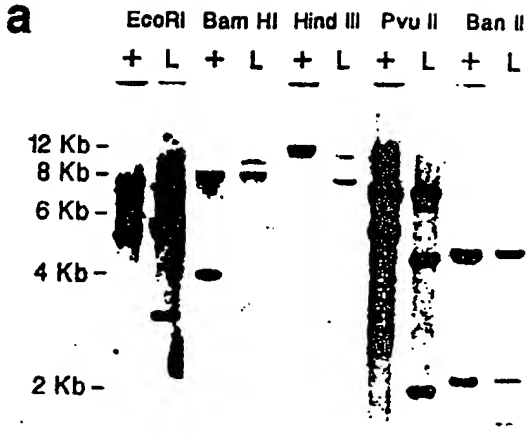

a
EcoRI
Bam HI
Hind III
Pvu II
Ban II
+ L + L + L + L + L
12 Kb
8 Kb
6 Kb
4 Kb
2 Kb

Fig. 1A

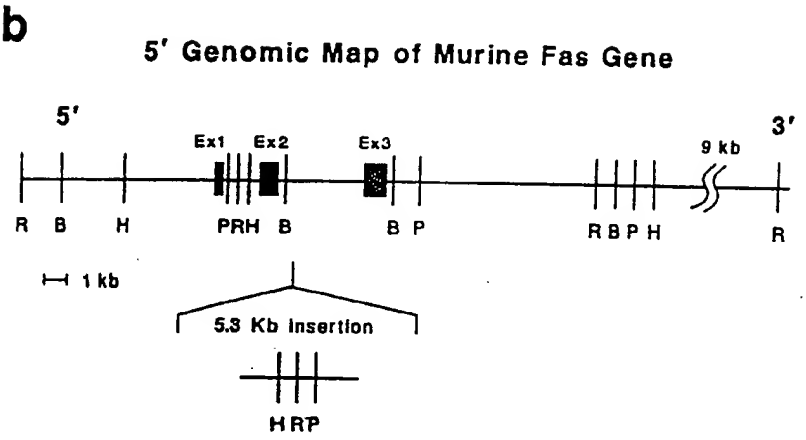

b
5' Genomic Map of Murine Fas Gene
5'
3'
Ex1
Ex2
Ex3
9 kb
R B H
PRH B
B P
R B P H
R
1 kb
5.3 Kb Insertion
HRP

Fig. 1B

Fig. 2A

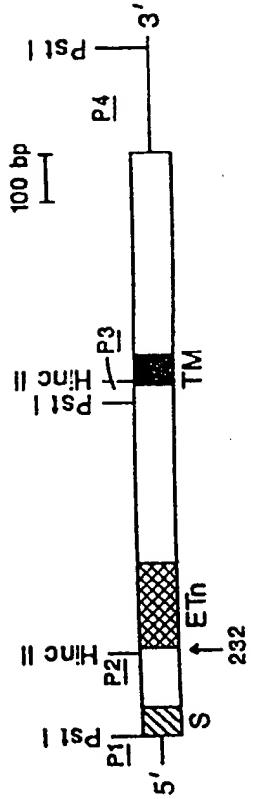
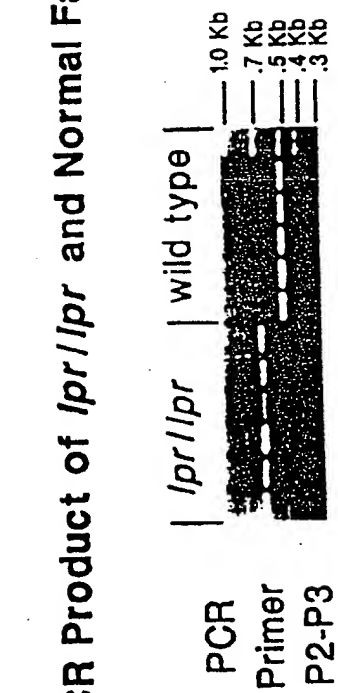
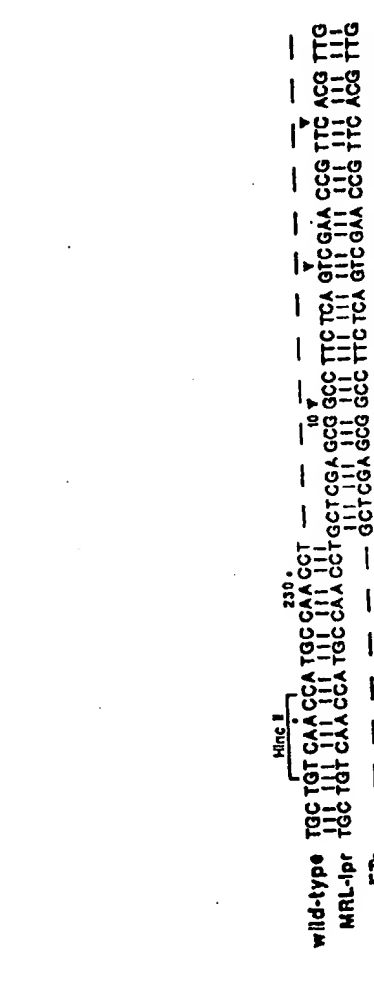

a
Pst I
3'
P4
100 bp
Hinc II
Pst I
P3
TM
Hinc II
P2
ETn
232
S
Pst I
P1
5'

Fig. 2B


b
PCR Product of lpr/lpr and Normal Fas
lpr/lpr
wild type
1.0 Kb
.7 Kb
.5 Kb
.4 Kb
.3 Kb
PCR Primer P2-P3


Nhe I
230
10
wild-type TGC TGT CAA CCA TGC CAA GCT — — — — — — — — — — — — — — —
MRL-lpr TGC TGT CAA CCA TGC CAA CCT GCT CGA GCG GCC TTC TCA GTC GAA CCG TTC ACG TTG
ETh — — — — — — — GCTCGA GCG GCC TTC TCA GTC GAA CCG TTC ACG TTG
amino acid CYS CYS GLN PRO CYS GLN PRO ALA ARG ALA ALA PHE SER VAL GLU PRO PHE THR LEU
wild-type —
MRL-lpr CGA GCT GCT GGC GGC CGC AAC ATT TTG GCG CCT GAA CAG GGA CCT GAA GAA TGG CAG
ETh CGA GGT GCT -GC GGC CGC AAC ATT TTG GCG CCg GAA CAG GGA CCT GAA GAA TGG CAG
amino acid ARG ALA ALA GLY GLY ARG ASN ILE LEU ALA PRO GLU GLN GLY PRO GLU GLU TRP GLN
wild-type —
MRL-lpr AGA GAT GCT AAG AGG AAC GCT GCA TTG GAG CTC CAC AGG AAA GGA TCT TCG TAT CGG
ETh AGA GAT GCT AAG AGG AAC GCT GCA TTG GAG CTC CAC AGG AAA GGA TCT TCG TAT CGG
amino acid ARG ASP ALA LYS ARG ASN ALA ALA LEU GLU LEU HIS ARG LYS GLY SER SER TYR ARG
240
wild-type — — — — — — GGT AAA AAA AAA GTT GAG GAC TGC AAA
180
MRL-lpr ACA TCG GAG CAA CGG ACA GGT AAA AAA AAA GTT GAG GAC TGC AAA
ETh ACA TCG GAG CAA CGG ACA — — — — — — — — —
amino acid THR SER GLU GLN ARG THR GLY LYS LYS LYS VAL GLU ASP CYS LYS

C

Fig. 2C

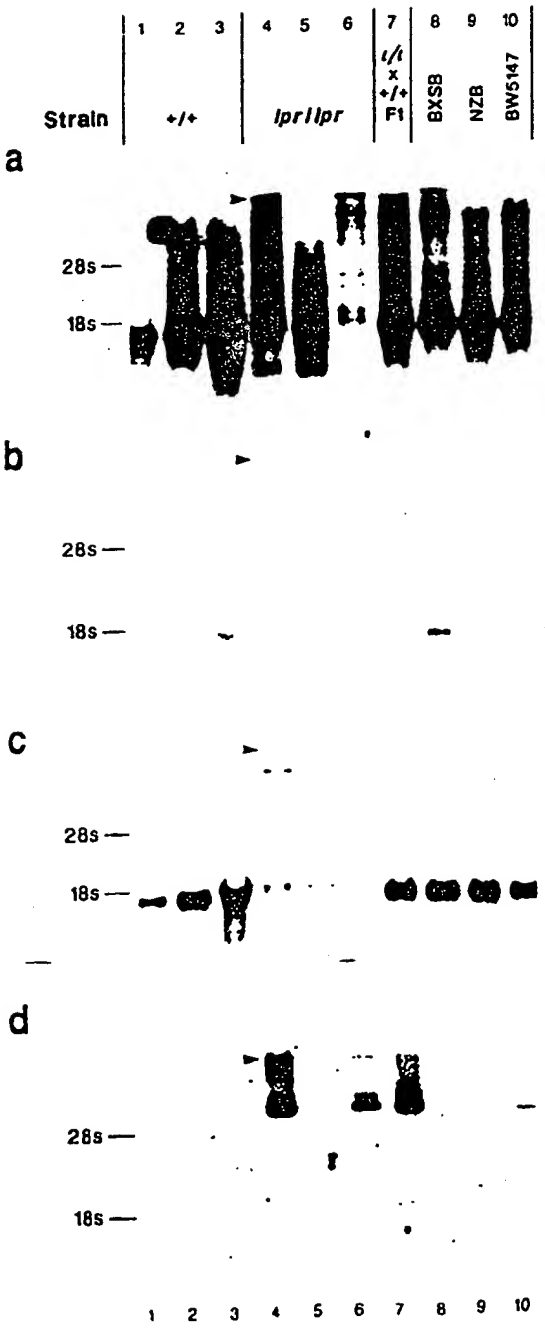


Fig. 3A

Fig. 3B

Fig. 3C

Fig. 3D

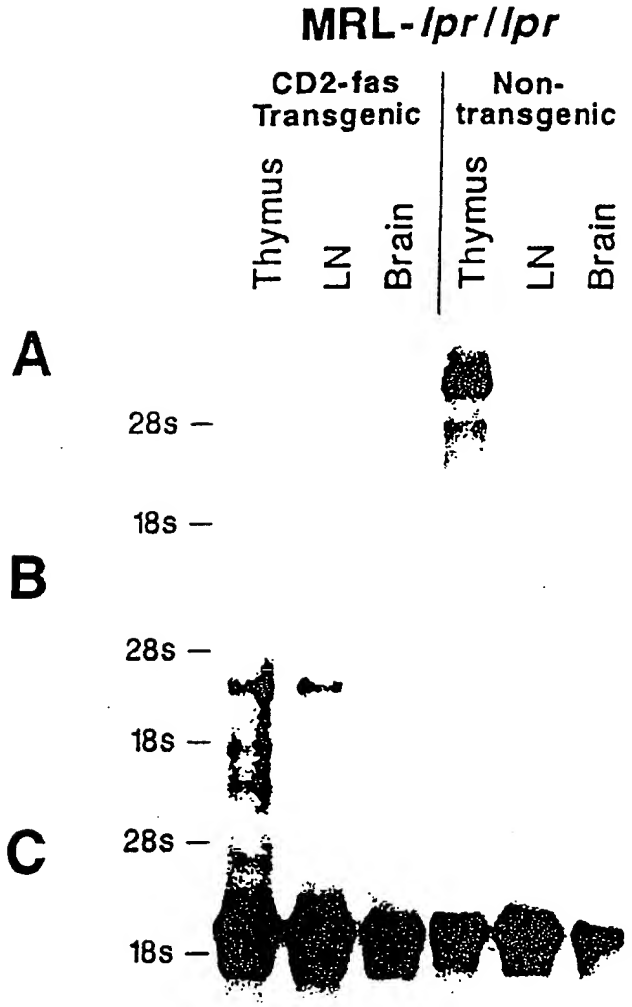


Fig. 4A

Fig. 4B

Fig. 4C

INTERNATIONAL SEARCH REPORT

In..ational application No.
PCT/US93/09839

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A01N 1/00; C07H 5/00; C07K 7/00, 13/00, C12Q 1/00
US CL :435/4, 6, 172.3; 514/44; 536/23.1, 23.4, 23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 6, 172.3; 514/44; 536/23.1, 23.4, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NATURE, Volume 356, issued 26 March 1992, R. Watanabe-Fukunage et al., "Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis", pages 314-317, see entire article.	1-23 and 26-27
Y	GENE, Volume 86, issued 1990, B.E. Shell et al., "Two subfamilies of murine retrotransposon ETn sequences", pages 269-274, see entire article.	1-23 and 26-27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 30 December 1993	Date of mailing of the international search report 18 JAN 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer BRIAN R. STANTON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09839

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, Volume 90, issued March 1993, M. Adachi et al., "Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of lpr mice", pages 1756-1760, see entire article.	1-23 and 26-27
Y	MOLECULAR AND CELLULAR BIOLOGY, Volume 7, number 4, issued April 1987, B. Shell et al., "Interruption of two immunoglobulin heavy-chain switch regions in murine plasmacytoma P3.26Bu4 by insertion of retroviruslike element ETn", pages 1364-1370, see entire article.	1-23 and 26-27
Y	CELL, Volume 66, issued 26 July 1991, N. Itoh et al., "The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis", pages 233-243, see entire article.	1-23 and 26-27
Y	THE JOURNAL OF IMMUNOLOGY, Volume 146, number 9, issued 01 May 1991, A.M. Krieg et al., "Association of murine lupus and thymic full-length endogenous retroviral expression maps to a bone marrow stem cell", pages 3002-3005, see entire article.	1-23 and 26-27
Y	THE JOURNAL OF IMMUNOLOGY, Volume 147, number 2, issued 15 July 1991, T. Zhou et al., "Abnormal thymocyte development and production of autoreactive T cells in T cell receptors transgenic autoimmune mice", pages 466-474, see entire article.	1-23 and 26-27
Y	HUMAN GENE THERAPY, Volume 2, issued 1991, F.D. Ledley, "Clinical considerations in the design of protocols for somatic gene therapy", pages 77-83, see entire article.	26-28
Y	EUROPEAN JOURNAL OF BIOCHEMISTRY, Volume 208, issued 1992, K. Roemer et al., "Concepts and strategies for human gene therapy", pages 211-225, see entire article.	26-27
Y	JOURNAL OF REPRODUCTIVE MEDICINE, Volume 37, number 6, issued June 1992, E.M. Karson et al., "Prospects for human gene therapy", pages 508-514, see entire article.	26-27
Y	THE EMBO JOURNAL, Volume 8, number 12, issued 1989, M. Cotten et al., "Ribozyme mediated destruction of RNA in viv ", pages 3861-3866, see entire article.	26-27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09839

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENTIFIC AMERICAN, Volume 263, number 5, issued November 1990, I.M. Verma, "Gene Therapy", pages 68-84, see entire article.	26-27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09839

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Databases: APS, ANABSTR, AQUASCI, BIOBUISINESS, BIOSIS, CA, CABA, CAPREVIEWS, CEBA, CEN, CIN, CJACS, CJELSEVIER, CONFSCI, DISSABS, EMBASE, FSTA, GENBANK, HEALSAFE, JICST-E, JPNEWS, LIFESCI, MEDLINE, NTIS, OCEAN

search terms: FAS; APOPTOS?; RETROVIR?; MOUNTZ?/AU; WU?/AU; ETN; TRANSPOS?; MRL; LPR

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I: Claims 1-21, drawn to DNA compositions and the first appearing method of use of said compositions, classified in Class 536, Subclass 23.5 and Class 435, subclass 6.

Group II: Claims 22-23, drawn to second methods of assaying biologically effective compounds, classified in Class 435, subclass 4.

Group III: Claims 26-27, drawn to third methods of treating autoimmune disease, classified in Class 514, subclass 44.

The three groups of inventions are distinct, one from the other, because they are drawn to materially different methods. In the case of group I, the method is drawn to the identification of particular populations of cells whereas in the methods of group II, the methods are directed towards the identification of particular bioeffective agents. These two different types of methods require separate and distinct considerations. For example, the methods of group I require consideration of the isolation and characterization of particular cellular populations whereas in the invention of group II, bioeffective agents must be taken into consideration.

The inventions of groups I and II are distinct from the therapeutic method of group III because therapeutic methods require consideration of routes of administration, targeting of therapeutic agents, assays for determination of efficacy, etc. Such considerations are not required for the analysis of either of the methods of groups I or II.

For the reasons stated above, the separate inventions lack any special technical feature linking the groups within the meaning of PCT Rule 13.2 and therefore lack unity of invention. Note that PCT Rule 13 does not provide for multiple methods within a single application.